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ORIGINAL ARTICLE

The notch pathway positively regulates programmed cell death during erythroid differentiation

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Programmed cell death plays an important role in erythropoiesis under physiological and pathological conditions. In this study, we show that the Notch/RBPjκ signaling pathway induces erythroid apoptosis in different hematopoietic tissues, including yolk sac and bone marrow as well as in murine erythroleukemia cells. In RBPjκ$^{-/-}$ yolk sacs, erythroid cells have a decreased rate of cell death that results in increased number of Ter119+ cells. A similar effect is observed when Notch activity is abrogated by incubation with the γ-secretase inhibitors, DAPT or L685,458. We demonstrate that incubation with Jagged1-expressing cells has a proapoptotic effect in erythroid cells from adult bone marrow that is prevented by blocking Notch activity. Finally, we show that the sole expression of the activated Notch1 protein is sufficient to induce apoptosis in hexametilene-bisacetamide-differentiating murine erythroleukemia cells. Together these results demonstrate that Notch regulates erythroid homeostasis by inducing apoptosis.
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Introduction

Notch is a highly conserved signaling pathway that regulates cell fate specification during development and adult tissue homeostasis. Physiological activation of the Notch pathway requires the interaction between the Notch receptor and one of its ligands. This interaction leads to the cleavage of Notch receptor, releasing the intracellular domain that translocates to the nucleus to bind RBPjκ and activate specific gene transcription (reviewed by Bray[1] and Lai[2]).

Notch function is required for the generation of definitive hematopoiesis as shown by the lack of hematopoietic precursors in the aorta of Notch1$^{-/-}$ and RBPjκ$^{-/-}$ mouse embryos or in mind bomb mutants in zebrafish.[3–6] In contrast, primitive hematopoiesis occurs in different Notch pathway mutants in both mouse and zebrafish.[3–5] In the mouse, primitive hematopoiesis originates in the blood islands of the yolk sac, starting at embryonic day 7.5 (E7.5). The main component of this primitive hematopoiesis is erythroid progenitor cells (EryP)[7] that generate large nucleated primitive erythrocytes that contain embryonic globins (βH1, ε-globin and ζ-globin) (reviewed by Palis and Segel[8]).

Erythropoiesis involves the progressive differentiation of uncommitted progenitors to mature erythrocytes. However, not only differentiation but also apoptosis participates in the regulation of cell survival and mature red cell turnover. The amount of erythropoietin (Epo), mainly dependent on hypoxia, is one of the key factors in controlling the survival of erythroid cells (reviewed in Mulcahy[9]). Expression of the antiapoptotic members of the Bcl-2 family, Bcl-2 and Bcl-x,[10] are some of the downstream effects of EpoR activation in this system. Consistent with this, bcl-x$^{-/-}$ embryos die of massive apoptosis in the nervous system and in fetal liver erythroid cells.[11]

Other transcription factors including GATA1 and, more recently, p53 have been implicated in regulating apoptosis at different stages of erythroid maturation.[12,13] In this sense, GATA1 plays a key role in development and survival of erythroid cells since GATA1-deficient cells failed to develop beyond the proerythroblast stage and undergo rapid apoptosis.[14]

Notch pathway has previously been shown to induce apoptosis in cell lines from different hematopoietic lineages most likely through the activation of its target gene hes1.[15–17] However, the overall data linking Notch and erythroid apoptosis is controversial.[18,19]

In this work, we demonstrate that the Notch signaling pathway is a positive regulator of apoptosis in primitive erythropoiesis in the yolk sac but also in erythroid cells from adult bone marrow (BM). Complementary studies using the murine erythroleukemia (MEL) cell line indicate that Notch induces erythroid-specific apoptosis.

Materials and methods

Animals

RBPjκ$^{-/-}$ mice have been described previously.[20] Animals were kept under pathogen-free conditions and experiments approved by the Animal Care Committee. Yolk sacs were obtained from timed pregnant females at days 7.5–9.5 of gestation and dissected out from embryo and vitelline arteries. Embryos were genotyped by polymerase chain reaction (PCR) and morphology. BM was obtained from 8 to 12-week wild-type (WT) CD1 mice.

Cell lines and transfections

MEL cells[21] were maintained in RPMI 10% fetal bovine serum (FBS), 1% L-glutamine and 1% Pen/Strep. Stable clones of MEL cells expressing N1ΔE[22] or pcDNA.3 were obtained by electroporation and expression was confirmed by western blot (9E10 antibody). NIH-3T3 cells were transfected by calcium

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phosphate with Jagged1 construct[23] and clones overexpressing Jagged1 were selected in G418. Differentiation of MEL cells was performed with 5 mM hexametilene-bisacetamide (HMBA; Sigma, St Louis, MI, USA) for 6 days.

Dianisidine staining

O-dianisidine (Sigma) was used to stain hemoglobin of both E9.5 WT and RBPjκ-mutant yolk sacs, and MEL friend cells to assay erythroid differentiation as described previously.[21]

Hematopoietic colony assay

Yolk sac from WT and RBPj$\kappa^{-/-}$ E7.5–9.5 embryos was digested in 0.1% collagenase (Sigma) in phosphate-buffered saline (PBS), 10% FBS for 30 min at 37°C. Cells (30 000) were plated in duplicates in 1% methylcellulose (Stem Cell Technologies, Vancouver, Canada) plus Iscove's with 10% FBS, 10% IL3- and stem cell factor (SCF)-conditioned medium, 2.5% L-glutamine, 0.1% monothioglycerol (Sigma), 1% Pen/Strep (Biological Industries, Beit Haemek Kibbutz, Israel), 2 IU/ml erythropoietin (Laboratorios Pensa-Esteve, Barcelona, Spain), 20 ng/ml granulocyte-macrophage colony-stimulating factor (PeproTech, Rocky Hill, NJ, USA) and 100 ng/ml of granulocyte colony-stimulating factor (Avantis Pharma, Paris, France). After 7 days, the presence of hematopoietic colonies was scored under a microscope. EryP colonies were scored at day 3.

Flow cytometry analysis

Collagenase-disrupted yolk sac cells were stained with fluorescein isothiocyanate FITC-conjugated CD71, CD41, ckit, CD45, and mac1 and PE-conjugated Ter119 and CD31 antibodies (Pharmingen, BD Biosciences, San Jose, CA, USA) or isotopic immunoglobulin G as a control. Cells were analyzed in a FACScalibur (Becton & Dickinson, BD Biosciences, San Jose, CA, USA) and WinMDI 2.8 software. Dead cells were excluded by 7-aminoactinomicin-D (7-AAD; Invitrogen, Carlsbad, CA, USA) staining. For the AnnexinV binding analysis, cells were stained with rh AnnexinV-FITC kit (Bender Medsystems, Burlingame, CA, USA) and 7-AAD for 15 min according to the manufacturer's instructions.

For cell cycle analysis of total yolk sac, cells were fixed in 70% EtOH at –20°C overnight, treated with 50 μg DNAse-free RNAse and stained with 25 μg of propidium iodide (Sigma). Ter119+ cell-cycle analysis was performed on fresh cells with 20 μM Draq5 (Biostatus Ltd, Leicestershire, UK). FlowJo 6.4.1 software was used for cell-cycle analysis.

Yolk sac and BM cultures

Collagenase-disrupted yolk sacs were cultured for 6 days in Iscove's with 10% FBS, 10% IL3- and SCF-conditioned medium, 0.1% monothioglycerol in the presence of 50 μM N-S-phenylglycine-t-butylester (DAPT) (Invitrogen), 2 μM L685,458 (Sigma) or dimethyl sulfoxide (DMSO) as control. For BM culture, 1.5×10^5 whole BM cells were incubated with γ-secretase inhibitors in RPMI 10% FBS, 2 IU/ml EPO for 2–3 days. Z-Val-Ala-DL-Asp-fluoromethylketone (Z-VAD-FMK; Bachem, Budendorf, Switzerland) was used at 200μM.

Coculture on 3T3 or 3T3-Jag1 stromal cells was perfomed with 4×10^5 whole BM cells in RPMI, 10% FBS for 16 h. Cells were assayed for AnnexinV binding and analyzed by flow cytometry.

Immunohistochemisty

Yolk sacs were fixed with 4% paraformaldehyde (Sigma), embedded in Paraplast (Sigma) and sectioned (10 μm). Slides were dewaxed in xylene, antigen retrieval was performed by boiling for 2 min in sodium acetate, rehydrated and blocked-permeabilized in 10% FBS, 0.3% Surfact-Amps × 100 (Pierce, Aalst, Belgium) and 5% non-fat milk in PBS for 90 min at 4°C. Anti P-Ser10 H3 (Upstate, Charlottesville, VA, USA) was used at 1:500 dilution and developed with Dakocytomation kit (Dako, Glostrup, DK, Denmark) following manufacturer's instructions. Hematoxilin (Merck, Whitehouse Station, NJ, USA) was used for counterstaining.

For histological analysis, tissue samples were fixed overnight at 4°C in 4% paraformaldehyde, dehydrated and embedded in Paraplast (Sigma). Samples were sectioned in a Leica-RM2135 at 4 μm and stained with hematoxilin and eosin.

Images were acquired with an Olympus BX-60 using a Spot camera and Spot 3.2.4 software (Diagnostic Instruments, Sterling Heights, MI, USA). Adobe Photoshop 6.0 software was used for photograph editing.

Semiquantitative reverse transcriptase-polymerase chain reaction

Total RNA from subdissected E9.5 WT and RBPj$\kappa^{-/-}$ yolk sacs was isolated using TRIzol Reagent (Invitrogen). Poly-AT Tract System IV (Promega, Madison, WI, USA) and RT-First Strand cDNA Synthesis Kit (GE Healthcare, Buckinghamshire, UK) were used to obtain mRNA and cDNA respectively. PCR product was analyzed at different cycles to avoid saturation. Quantity One software (Biorad, Hempstead, UK) was used for densitometry. Primer pairs used in the experiments are listed in Supplementary Table S1.

Quantitative RT-PCR

Ter119+ cells were sorted from E9.5 collagenase-treated embryos in MoFlo cell sorter (Dakocytomation, BD Diagnostic, San Jose, CA, USA). mRNA was isolated with Rneasy minikit (Qiagen, Valencia, CA, USA) following manufacture's instructions. qRT-PCRs were performed with SYBR Green I Master (Roche, Basel, Switzerland) in LightCycler480 system.

Statistical analysis

Normal distribution of the samples was confirmed with one-sample Kolmogorow–Smirnoff test and Student's *t*-test was performed.

Results

Absence of Notch signaling results in increased number of erythroid cells in the yolk sac

Notch signaling has previously been shown to influence differentiation and apoptosis of erythroid cells *in vitro* although controversial observations have been reported.[15,18,19,24] For this reason, we aimed to characterize the physiological role of the Notch pathway in erythropoiesis by comparing WT and RBPj$\kappa^{-/-}$ embryos. Despite the absence of intraembryonic hematopoiesis in the RBPj$\kappa^{-/-}$ embryos and the presence of different angiogenic abnormalities in the yolk sac, we found that primitive hematopoiesis does occur in the yolk sac of the RBPj$\kappa^{-/-}$ embryos (Figure 1a), similar to the Notch1$^{-/-}$ mutants.[25–27]

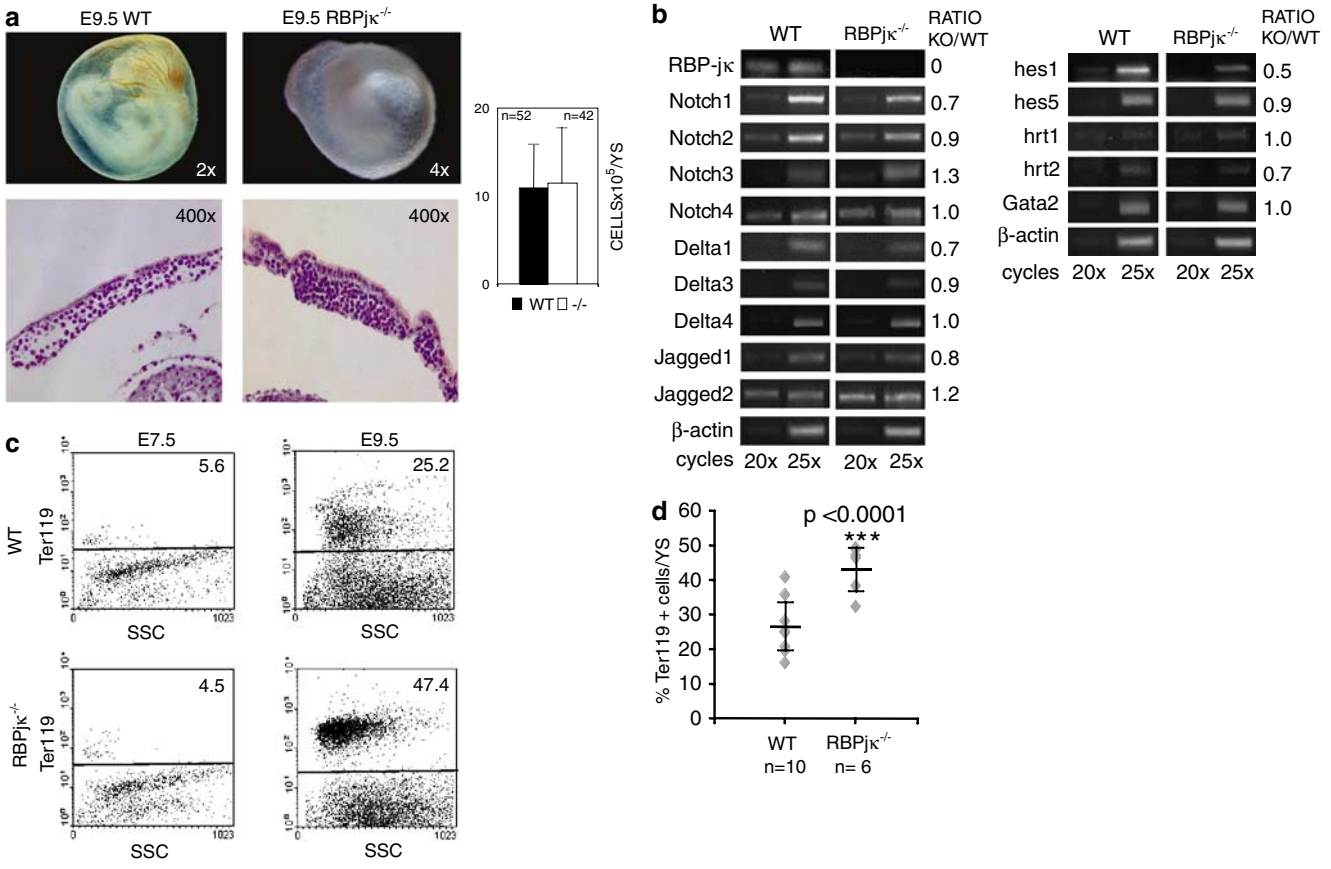

Figure 1 Increased number of erythroid cells in RBPj$\kappa^{-/-}$ yolk sacs. (**a**) Images of E9.5 WT and RBPj$\kappa^{-/-}$ embryos with the yolk sac (upper panel) and hematoxilin/eosin staining of yolk sac sections. Total number of cells obtained from disrupted yolk sacs (right panel). (**b**) Semiquantitative RT-PCR of Notch receptors, ligands and target genes from E9.5 WT and RBPj$\kappa^{-/-}$ yolk sacs. (**c**) Representative analysis of Ter119+ cells from E7.5 and E9.5 WT and RBPj$\kappa^{-/-}$ yolk sacs. (**d**) Percentage of Ter119+ cells in the analyzed E9.5 WT and RBPj$\kappa^{-/-}$ yolk sacs. Average and s.d. are represented.

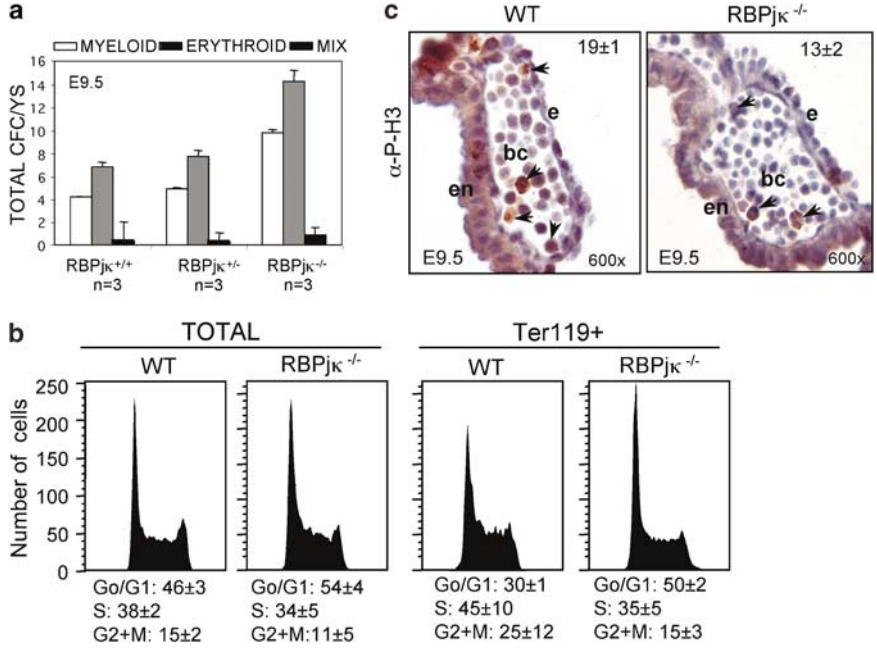

Figure 2 Proliferation in E9.5 WT and RBPj$\kappa^{-/-}$ yolk sac cells. (**a**) Graphs represent the total number of CFC types obtained from WT or RBPj$\kappa^{-/-}$ yolk sacs. (**b**) Representative cell-cycle analysis from total and Ter119+ cells from E9.5WT or RBPj$\kappa^{-/-}$ yolk sacs. Average values and s.d. from two yolk sacs are shown. (**c**) IHC of P-Histone3 in yolk sac sections. Numbers represent the average percentage of positive cells inside the blood islands found in four independent stainings. Arrowheads indicate cells with positive staining; e, endothelium; en, endoderm; bc, blood cells.

To determine whether Notch pathway plays a role in regulating hematopoiesis in the yolk sac, we first analyzed the expression of different Notch receptors, ligands and Notch-target genes in the yolk sac of WT and RBPj$\kappa^{-/-}$ embryos by semiquantitative RT-PCR and we observed that all Notch family genes are expressed in the yolk sac at E9.5 (Figure 1b). In RBPj$\kappa^{-/-}$, we found reduced expression of all ligands and receptors, whereas Notch3 and Jagged2 were upregulated. We also tested the expression of Notch-target genes and detected a consistent reduction in hes1 levels in RBPj$\kappa^{-/-}$ yolk sacs compared with WT (Figure 1b).

Since hematopoiesis in the yolk sac is mainly restricted to erythropoiesis, we determined the percentage of cells expressing the erythroid marker Ter119 in collagenase-treated yolk sacs at E7.5, E8.5 and E9.5. We detected a few positive cells in the yolk sac of E7.5 (Figure 1c) and E8.5 (data not shown). At day 9.5, the percentage of Ter119+ in the yolk sac ranged from 20 to 40% in the WT and 40–60% in the RBPj$\kappa^{-/-}$ (Figure 1c and d) being similar the total number of cells per yolk sac (Figure 1a).

Proliferation is not responsible for increased erythropoiesis in RBPj$\kappa^{-/-}$ yolk sacs

To investigate whether the higher number of Ter119+ cells in the RBPj$\kappa^{-/-}$ yolk sac was owing to an increase in the number of progenitors, we performed colony-forming cell (CFC) assays with collagenase-treated yolk sac cells at E7.5, E8.5 and E9.5 from RBPj$\kappa^{+/+,+/-}$ and $^{-/-}$ embryos. We detected a similar percentage of myeloid, erythroid and mixed colonies in these cultures (Supplementary Figure S1), however; at E9.5, there was a twofold increase in the total number of CFC in mutant embryos (Figure 2a). To test whether this effect was due to increased proliferation, we analyzed the cell-cycle profile of WT and RBPj$\kappa^{-/-}$ yolk sac cells by flow cytometry. Surprisingly, the percentage of cells in S/G_2–M phase was slightly reduced in RBPj$\kappa^{-/-}$ compared with WT cells (from 53 to 45%), and this reduction in S/G_2–M phase was higher when cell cycle was analyzed in the Ter119+ cells (Figure 2b). To confirm this observation, we performed the P-H3 staining on yolk sac sections to assess the number of cells undergoing mitosis inside the blood islands. P-H3 staining showed that hematopoietic

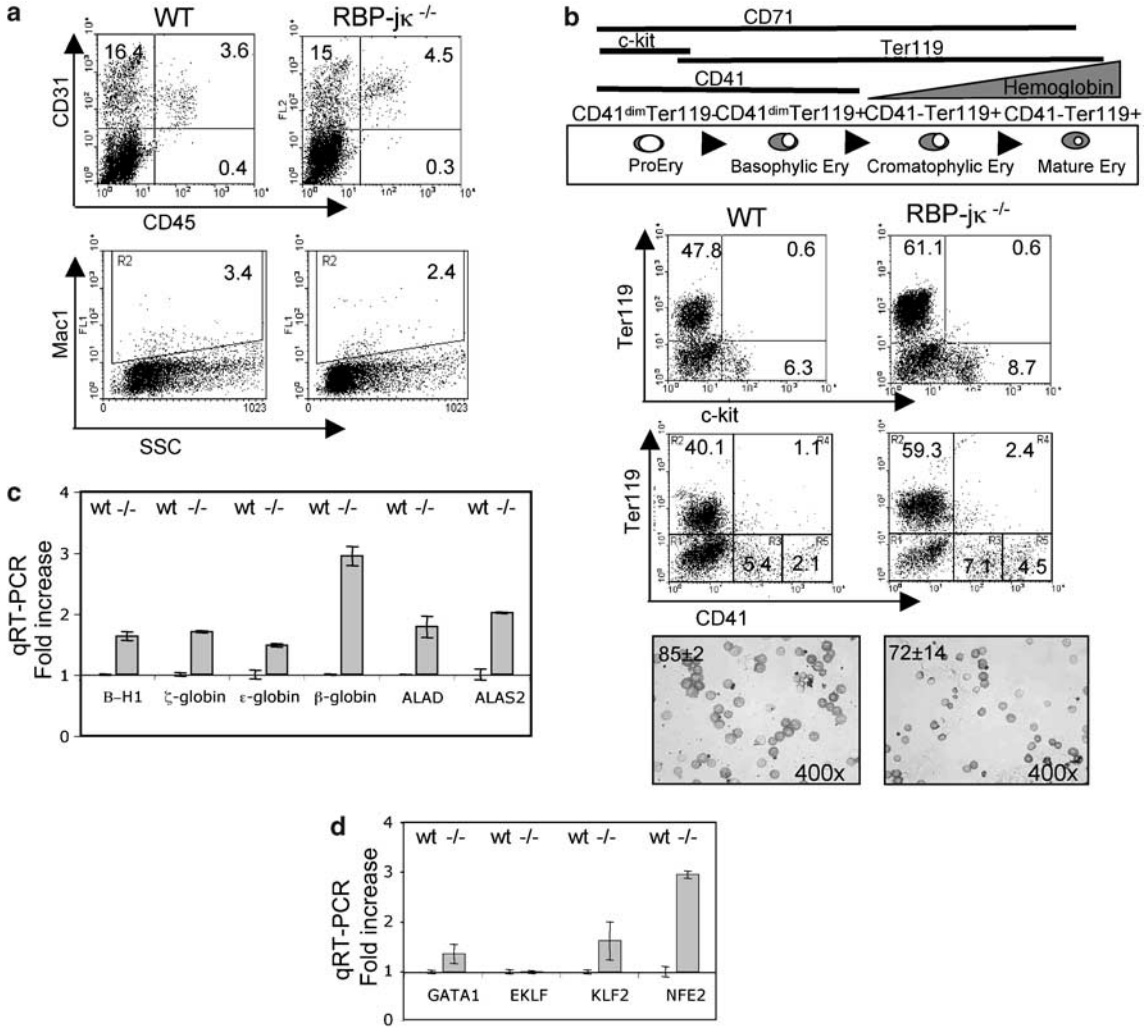

Figure 3 Erythroid differentiation is not impaired in RBPj$\kappa^{-/-}$ yolk sacs at E9.5. (**a**) Representative dot plots showing expression of CD45 (hematopoietic marker) versus CD31 (endothelial marker) and mac1 (macrophage marker). (**b**) Representation of erythroid differentiation markers (upper). Dot plots of representative erythroid subpopulations from three WT and RBPj$\kappa^{-/-}$ yolk sacs. Dianisidine staining of circulating cells from the yolk sac (lower panels). Numbers represent the average and s.d. of positive cells counted in three different samples. (**c** and **d**) qRT-PCR of (**c**) globin genes and hemo maturation enzime genes and (**d**) erythroid-specific transcription factors from WT and RBPj$\kappa^{-/-}$ yolk sacs.

cells from the WT yolk sacs have a similar mitotic rate (19.8 %) than cells in RBPj$\kappa^{-/-}$ yolk sacs (12.9 %); (Figure 2c). Altogether these results indicate that proliferation is not increased in the RBPj$\kappa^{-/-}$ yolk sac erythroid cells.

Normal differentiation occurs in RBPjκ$^{-/-}$ yolk sac

Although hematopoiesis is mainly restricted to erythropoiesis in the yolk sac, different progenitor types and macrophages are also generated. As we detected increased number of different hematopoietic progenitors in the RBPj$\kappa^{-/-}$ (Figure 2a), we speculated that the decision between hematopoietic and endothelial lineages may be affected. To test this possibility, we analyzed the expression of CD45 (hematopoietic excluding erythroid cells) and CD31 (endothelial) cell markers. We detected a similar number of cells expressing these markers, indicating that the non-erythroid hematopoiesis is normally occurring in the RBPj$\kappa^{-/-}$ yolk sac (Figure 3a). Consistent with this observation, we did not detect any difference in the percentage of endothelial cells (CD31+ and CD45−; Figure 3a) or in the expression of PECAM or VE-cadh genes in these yolk sacs (Supplementary Figure S4), in contrast to that previously observed in the intraembryonic endothelial/hematopoietic differentiation[3,6] In addition, no major differences were found in the percentage of mac1+ cells between WT and RBPj$\kappa^{-/-}$ (Figure 3a).

We next investigated whether the higher number of Ter119+ cells in the RBPj$\kappa^{-/-}$ was due to a blockage in erythroid differentiation. Thus, we characterized the different erythroid subpopulations by analyzing the expression of specific differentiation markers CD71, CD41 and c-kit in the Ter119+ population by flow cytometry. As shown in Supplementary Figure S2, CD71 was expressed in all Ter119+ cells in both WT and RBPj$\kappa^{-/-}$ yolk sacs, this result is surprising since this marker is downregulated during erythroid differentiation in BM.[28] Analysis of other differentiation markers showed that the different Ter119+ subpopulations were similarly represented in WT and mutant yolk sacs (Figure 3b). We also analyzed the expression of erythroid transcription factors and globin genes in purified Ter119+ cells from WT and RBPj$\kappa^{-/-}$ embryos by qRT-PCR. We did not detect major differences in the expression of the embryonic globins and hemo group maturation enzymes (ALAD and ALAS2; Figure 3c) or in the percentage of circulating yolk sac cells showing dianisidine staining (from 85% to 72%; Figure 3b); however, a threefold increase in the expression of adult β-globin was observed in the RBPj$\kappa^{-/-}$ cells compared with the WT (Figure 3c). We also detected overexpression of the erythroid transcription factor NFE2 in RBPj$\kappa^{-/-}$ erythroid cells, whereas no significant differences were detected in GATA1, KLF2 and EKLF levels (Figure 3d). Surprisingly, we did not observed downregulation of the hes1 gene in the Ter119+ cells of the RBPj$\kappa^{-/-}$ embryos (data not shown).

Reduction of apoptosis in Notch-defective yolk sac erythroid cells

Apoptosis is a crucial mechanism for maintaining the homeostasis of the erythroid lineage. Since minor differences in

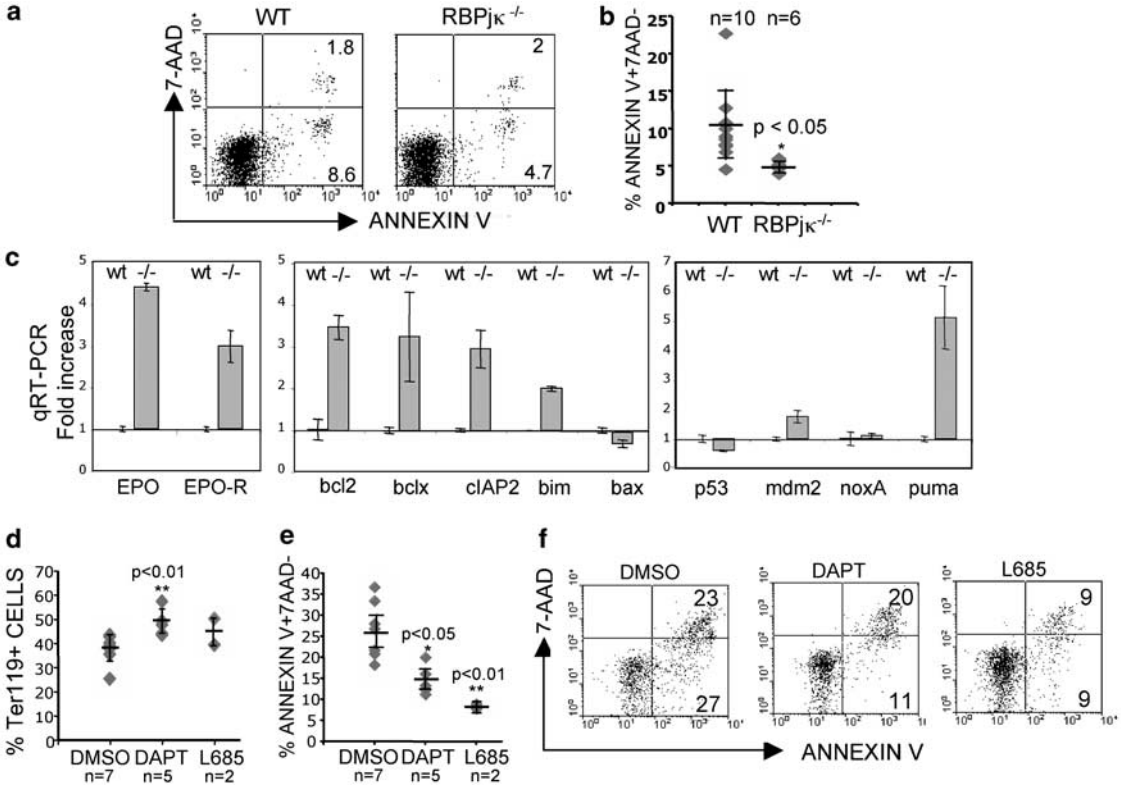

Figure 4 RBPj$\kappa^{-/-}$ and γ-secretase inhibitor-treated erythroid cells show reduced apoptosis. (**a**) Dot plot of AnnexinV and 7-AAD staining in a representative E9.5 WT and RBPj$\kappa^{-/-}$ yolk sac. (**b**) Percentage of AnnexinV + 7AAD– cells in the Ter119+ cells in E9.5 WT and RBPj$\kappa^{-/-}$ yolk sacs. (**c**) Relative expression of pro- and antiapoptotic genes in E9.5 RBPj$\kappa^{-/-}$ compared with WT yolk sacs by qRT-PCR. (**d** and **e**) Graphs represent the percentage of Ter119+ cells (**d**) and AnnexinV + 7AAD-cells (**e**) from E9.5 WT yolk sacs after 5 days of culture in DMSO, 50 μM DAPT or 2 μM L685,458. (**f**) Dot plots from a representative experiment.

proliferation or differentiation were found in the RBPj$\kappa^{-/-}$ mutants, we tested whether the increased number of erythroid cells in these embryos was due to differences in apoptosis. We detected a significant reduction ($P<0.05$) in the apoptotic rate as measured by AnnexinV binding in the RBPj$\kappa^{-/-}$ yolk sacs that was specific for the erythroid Ter119+ population (Figure 4a and b).

Next, we analyzed the expression levels of different genes that are associated with apoptosis in purified Ter119+ cells. By qRT-PCR, we detected 3–4-fold increase in the expression levels of the erythroid survival factor EPO, its receptor and its downstream effectors, the antiapoptotic *bcl-2* and *bcl-x* genes in the RBPj$\kappa^{-/-}$ cells compared with the WT. This suggests that EpoR-mediated signaling may be participating in the increased survival of RBPj$\kappa^{-/-}$ Ter119+ cells. In contrast, no major differences were found in the expression levels of *p53* family genes although one of its proapoptotic targets *puma* was increased in the mutant cells compared with the WT (Figure 4c, right panel).

To further demonstrate that reduced apoptosis in the RBPj$\kappa^{-/-}$ erythroid cells was dependent on Notch function, we obtained WT cells from disrupted yolk sacs and incubated in liquid culture in the presence or absence of the γ-secretase inhibitors DAPT and L685,458. After 6 days of culture, both the total number and the percentage of TER119+ cells was significantly increased in the DAPT-treated cells compared with the control (Figure 4d) resembling the RBPj$\kappa^{-/-}$ phenotype. The increase in the Ter119+ population correlated with a threefold reduction in the percentage of AnnexinV+ cells in the DAPT- and L685,458-treated cells (Figure 4e and f). In agreement with the differences in gene expression found in the RBPj$\kappa^{-/-}$ yolks sacs, upregulation of EPO, its receptor, *bcl-2* and *bcl-x* was detected in the γ-secretase-treated Ter119+ cells (Supplementary Figure S5). Together these results indicate that Notch activity regulates apoptosis in the erythroid lineage in the yolk sac.

Notch activation induces apoptosis in adult BM erythroid and MEL cells

We next investigated whether Notch activation was regulating apoptosis not only in the yolk sac but also in adult erythropoiesis. Since RBPj$\kappa^{-/-}$ mutants do not generate definitive hematopoiesis and die at E10.5, we isolated BM from WT adult mice and performed cell cultures in the presence of DMSO, DAPT or L685,458. We observed a 30% decrease in the percentage of AnnexinV+ cells in the Ter119+ population after 2 days of culture in γ-secretase inhibitors compared with the control (Figure 5a). Interestingly, Ter119-negative cells were not protected from apoptosis by DAPT in these cultures (data not shown), indicating the specific proapoptotic effect of Notch activity on the erythroid lineage. Conversely, incubation of total BM cells from adult mouse on NIH-3T3-Jag1 cells resulted in a

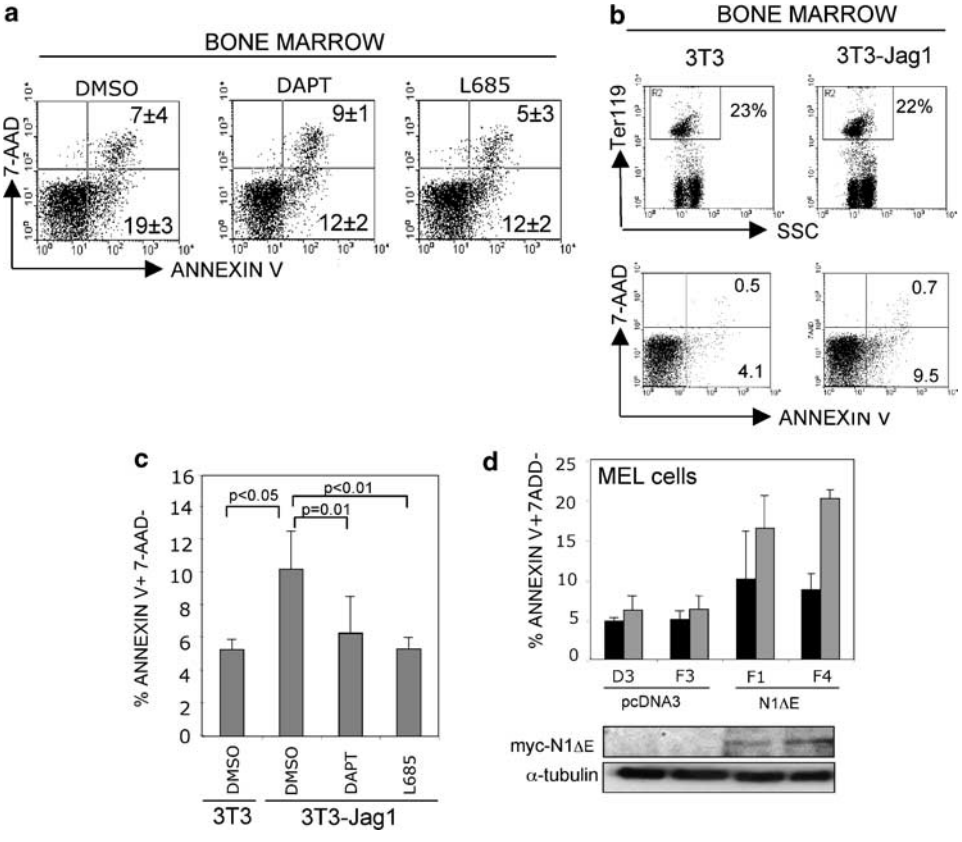

Figure 5 Notch activity induces apoptosis in the Ter119+ cells from adult bone marrow and murine erythroleukemia cells. (**a**) Graphs represent the percentage of AnnexinV+7AAD- in the Ter119+ population of BM cultured in the presence of DMSO, DAPT or L685,458. Average percentage and s.d. of three different experiments are shown. (**b** and **c**) Representative flow cytometry analysis of AnnexinV binding in the Ter119+ cells from BM incubated on 3T3 or 3T3-Jag1 for 16 h (**b**). Graphs represent the average and s.d. of 3T3-Jag1-induced apoptosis observed in three different experiments incubated with DAPT and L685,458 as measured by AnnexinV+7AAD- in Ter119+ cells (**c**). (**d**) Graphs represent the average percentage of AnnexinV+7AAD- cells in Notch1ΔE expressing MEL clones (F1, F4) compared with control clones (D3, F3) in proliferantion media (black bars) or after 6 days of differentiation in 5 mM HMBA (gray bars). Western blot shows the expression of Notch1ΔE.

2–3-fold increase in the percentage of AnnexinV + Ter119 + population compared with that incubated on NIH-3T3 control cells ($P = 0.04$) (Figure 5b and c). Moreover, this effect was prevented in the presence of γ-secretase inhibitors DAPT ($P = 0.01$) or L685,458 ($P = 0.002$; Figure 5c) and the caspase inhibitor Z-VAD-FMK (data not shown).

To further demonstrate that Notch was sufficient to induce apoptosis in erythroid cells, we generated different clones of MEL cells expressing the active Notch1 fragment, N1ΔE. Cells expressing N1ΔE differentiated and proliferated similar to the controls (Supplementary Figure S3); however, these cells showed a higher percentage of AnnexinV binding after 6 days in culture under differentiating conditions (HMBA) as expected (Figure 5d).

Altogether our results indicate that Notch positively regulates apoptosis in embryonic and adult erythropoiesis and in differentiating erythroleukemia cells.

Discussion

Notch signaling had been linked previously to regulate apoptosis in erythroid cells, although it was unclear whether Notch inhibits or promotes apoptosis. We have now used primary cells from yolk sac and BM to elucidate the physiological effect of Notch activation on the erythroid lineage. We demonstrate that the Notch pathway is a positive inducer of apoptosis in erythroid cells from different tissues.

Although different cytokines regulate the integrity of the erythroid compartment, production of definitive erythroid cells is mainly dependent on Epo.[29] Signaling from EpoR through Jak2/Stat5[30,31] prevents apoptosis on this cell lineage from late erythroid progenitors (CFU-E) until the onset of hemoglobinization,[32,33] and one important target gene of this cascade is bcl-x.[34] Studies using bcl-x-null mice demonstrated a critical role for this gene at the end of erythroid maturation when maximal hemoglobin synthesis occurs.[35] RBPj$\kappa^{-/-}$ erythroid cells show a clear upregulation of genes involved in this antiapoptotic pathway, such as EPO, EPO-R, bcl-x and bcl-2, suggesting that Notch may induce apoptosis by impinging on this pathway. Previous reports have attempted to link Notch induction of apoptosis through the p53 pathway in other systems.[36,37] We have also explored that possibility in erythroid cells, since there is increasing evidence of p53-mediated apoptosis at different stages of erythroid differentiation;[13,38] however, our results show that crucial p53 pathway genes are not affected in the RBPj$\kappa^{-/-}$ erythroid cells suggesting that this pathway is not responsible for the protection of apoptosis in the RBPjκ mutants. In fact, we found an erythroid-specific upregulation of the proapoptotic p53-target gene, *puma*, in the RBPj$\kappa^{-/-}$ Ter119 + cells, which is surprising since this population has a lower apoptotic rate.

Although previous reports have attempted to decipher which is the Notch function in apoptosis of erythroid cells,[15,19] this is the first time that this question is addressed in primary cells. The use of cell lines in the previous published reports may explain the controversial data. Our data is in agreement with the results from Ishiko *et al*.[15] using K562 cells in which Notch activity induces apoptosis. In this system, Notch activation led to downregulation of bcl-x, likely through repression of GATA1 by hes1 under differentiation conditions.[15] Similarly, we found increased levels of bcl-x and bcl-2 in the Ter119 + cell population of RBPj$\kappa^{-/-}$; however, we did not detect any change in hes1 and GATA1 levels in this model. The observation that hes1 is not affected by the lack of RBPjκ in particular cell types has already been described and explained by the repressor function of RBPjκ in the absence of Notch signaling (reviewed by Lai[2]).

We now have used different approaches including the Notch loss-of-function RBPj$\kappa^{-/-}$ embryos, γ-secretase inhibitors and Jag1-expressing cell co-culture to investigate the role of Notch signaling in regulating apoptosis on erythroid cells. Together, our results confirm that Notch activation favors apoptosis in this particular lineage and this finding may be particularly relevant in some erythroid disorders. In fact, erythropoiesis in the RBPj$\kappa^{-/-}$ embryos resembles the one observed in myelodysplastic syndrome (MDS)-derived leukemias or polycytemia vera. These human myeloproliferative syndromes cause the overproduction of erythroid cells, mainly because of activating mutations in the Jak2 kinase and this leads not only to increased cell proliferation but also upregulation of *bcl-x* (reviewed by Campbell and Green[39]) and protection from apoptosis. In this scenario and based on our results, it is particularly interesting to investigate whether the Notch pathway is deregulated in these tumors. In this sense, higher expression of the Notch-like ligand, dlk, in MDS compared with myeloid leukemia cells has been detected by microarray analysis.[40]

Notch activation has previously been associated to inhibition of apoptosis in normal endothelial[41] and T cells,[42] likewise in neoplastic cells including Kaposi's sarcoma,[43] glioma[44] and Hodgkin's lymphoma[45] cells. Conversely, Notch activation can induce apoptosis during the development of the retina[46] and the serotonin lineage[47] in *Drosophila*. Notch also induces apoptosis in B-cell lymphomas,[16] normal monocytes[48] or hepatocellular carcinoma cells.[49] Based on this and our results, it is likely that apoptosis is another mechanism used by Notch to control specific tissue homeostasis.

Altogether, our results indicate that the activation of the Notch pathway is able to regulate the erythroid lineage in different hematopoietic tissues by inducing apoptosis.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)